Dual mechanisms of posttranscriptional regulation of Tet2 by Let-7 microRNA in macrophages

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Tet methylcytosine dioxygenase 2 (Tet2) is an epigenetic regulator that removes methyl groups from deoxycytosine residues in DNA. Tet2-deficient murine macrophages show increased lipopolysaccharide (LPS)-induced and spontaneous inflammation at least partially because Tet2 acts to restrain interleukin (IL)-1β and IL-6 expression in induced cells. MicroRNAs have emerged as critical regulatory noncoding RNAs that tune immune cell responses to physiological perturbations and play roles in pathological conditions in macrophages. To determine if a microRNA played any role in Tet2 activity, we examined the interrelationship of Tet2 action and the let-7 microRNA family, utilizing several let-7 microRNA engineered murine models. We first showed that Tet2, but not Tet3, is a direct target of the let-7a-1/let-7d/let-7f-1 (let-7adf) microRNAs in macrophages. We found that overexpression or deletion of the let-7adf gene cluster causes altered IL-6 induction both in tissue culture cells induced by LPS treatment in vitro as well as in a *Salmonella* infection mouse model in vivo. Mechanistically, let-7adf promotes IL-6 by directly repressing Tet2 levels and indirectly by enhancing a Tet2 suppressor, the key TCA cycle metabolite, succinate. We found that Let-7adf promotes succinate accumulation by regulating the Lin28a/Sdha axis. We thereby identify two pathways of let-7 control of Tet2 and, in turn, of the key inflammatory cytokine, IL-6, thus characterizing a regulatory pathway in which a microRNA acts as a feedback inhibitor of inflammatory processes.

Tet2 | lipopolysaccharide | Let-7 | succinate | IL-6

Enzymes that act on epigenetic processes regulate the functioning of immune cells. Targeting of these enzymes has been proven to be a valuable tool to dampen inflammatory responses (1, 2). Tet2 is one of the earliest and most frequently mutated genes in myeloid neoplasms, including myelodysplastic syndrome and chronic myelomonocytic leukemia (3). Inactivating mutations in Tet2 can cause epigenetic dysregulation and monocytic lineage skewing (4, 5). Loss of Tet2 results in the up-regulation of interleukin (IL)-6 following a challenge with lipopolysaccharide (LPS) and an increase of IL-1β expression in macrophages (6, 7). Tet2-deficient mice are highly susceptible to dextran sulfate-induced colitis, with a more severe inflammatory phenotype and increased IL-6 production compared with WT control mice (6). However, there may be mechanisms of regulation of immunity and inflammation by Tet2 independent of its classical well-known role in modulating DNA demethylation.

MicroRNAs (miRs) are short noncoding RNAs of ∼22 nt that are involved in the complex posttranscriptional regulatory networks that control cellular functions such as inflammation and metabolism (8, 9). MiR let-7 clusters have been identified as an important contributor to many physiological and pathological processes, for instance, tumorigenesis and B cell antibody production (10–12). However, the role of miRs in regulating Tet gene expression remains unclear. Intriguingly, it has been recently reported that succinate, a TCA cycle intermediate, can inhibit Tet2 (13–16). IL-6 is a key mediator of inflammation. What remains obscure is the regulatory mechanisms that link succinate to the alteration of IL-6 levels in inflamed macrophages. Identifying whether and how a let-7 cluster might be involved in regulating IL-6 in macrophage inflammatory responses would add significantly to our understanding of inflammatory processes.

We herein report that the miR-let-7a/let-7d/let-7f cluster (let-7adf) is involved in promoting IL-6 by regulating Tet2 through two distinct mechanisms. We demonstrate that let-7adf contributes to LPS-driven metabolic reprogramming by activating both glycolysis and succinate accumulation, the latter a consequence of its ability to significantly reduce the amount of succinate dehydrogenase subunit A (Sdha) in activated cells by targeting Lin28a. We found that let-7adf enhances IL-6 in macrophages by repressing the expression of Tet2, and it also promotes IL-6 levels by increasing succinate accumulation through regulating the Lin28a/Sdha axis in LPS-activated macrophages.

Results

Tet2, but Not Tet3, Is a Direct Target of the Let-7adf Cluster in LPS-Activated Macrophages. We found that both Tet2 and Tet3 were predicted to be potential targets of let-7d (Fig. 1*A*, only Tet2 3′ UTR shown) by using computational prediction programs (17). To investigate if TET proteins might be regulated by let-7 in LPS-activated macrophages, we first examined TET protein and mRNA levels in bone marrow-derived macrophages (BMDMs) derived from let-7adf knockout (KO) and WT control mice. We found a significant increase in the TET2 protein level in the absence of let-7adf, compared with WT control BMDMs (Fig. 1*B*), while the expression of TET1 and TET3 were comparable

Significance

Macrophages function as sentinel cells, constantly monitoring the host environment for bacterial infection. They are activated by bacterial components and then secrete proinflammatory cytokines, such as interleukin (IL)-6. This is one of the body's most effective weapons for fighting infections but must be rigidly controlled to avoid pathologic overresponse. Many factors have been shown to act as feedback regulators of inflammatory processes. Using both knockout and myeloid cell-specific transgenic mouse models, we report here that a physiological role of the microRNA let-7adf cluster is to promote IL-6 secretion by lipopolysaccharide-activated macrophages through down-regulating Tet2. This microRNA cluster represses Tet2 using two different mechanisms: direct targeting and indirect enhancement of succinate accumulation, a compound that represses Tet2.

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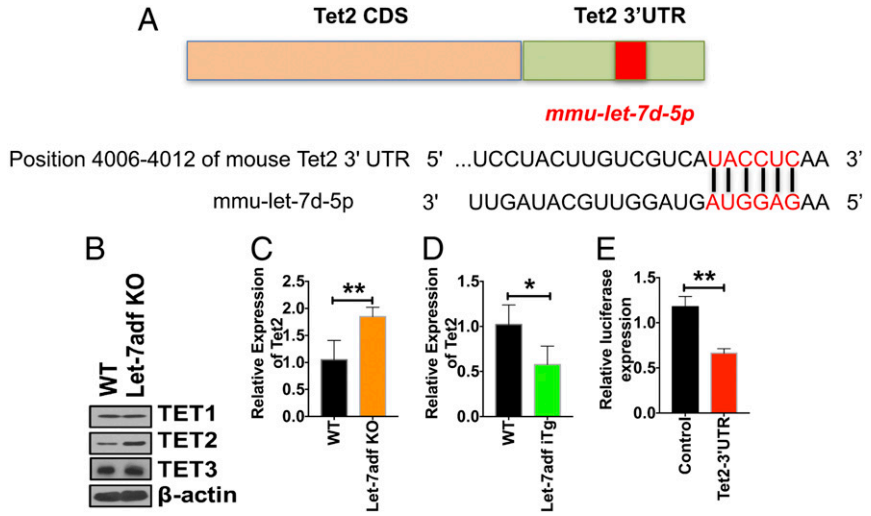

Fig. 1. Tet2 is a target of the let-7adf cluster in LPS-activated macrophages. (*A*) Schematic of the predicted let-7d binding site in the Tet2 3′UTR. (*B*) The protein expression of Tet1, Tet2, and Tet3 in BMDMs from WT or the let-7adf cluster KO mice obtained by Western blot. (*C*) RT-qPCR of the Tet2 mRNA level in WT control or the let-7adf cluster KO BMDMs. (*D*) RT-qPCR of Tet2 in WT control or the let-7adf cluster iTg BMDMs. (*E*) Tet2 3′UTR luciferase reporter assays in HEK293T cells. $^{**}P < 0.01$, $^{*}P < 0.05$, using a Student's *t* test.

between KO and WT BMDMs, implying that Tet2 might be an authentic target of let-7adf in macrophages (Fig. 1*B*). To investigate whether overexpression of let-7adf in macrophages has the opposite effect, we generated let-7adf myeloid-cell specific overexpression transgenic mice (denoted as let-7adf iTg mice). We observed a decrease of TET2 protein expression in the let-7adf iTg BMDMs compared with WT control BMDMs but again saw comparable levels of TET1 and TET3 protein (*SI Appendix*, Fig. S1). These results imply that let-7adf selectively inhibits TET2 protein expression in LPS-activated macrophages.

To further examine the direct effects of let-7adf on Tet2, we measured the mRNA level in LPS-activated let-7adf–deficient macrophages. We found that the Tet2 mRNA level was higher in the let-7adf KO BMDMs compared with WT BMDMs (Fig. 1*C*), while BMDMs overexpressing let-7adf displayed a decreased level of Tet2 mRNA, compared with WT BMDMs (Fig. 1*D*), confirming that let-7adf represses Tet2 mRNA in LPS-activated macrophages. Tet1 and Tet3 were unaffected (*SI Appendix*, Figs. S2 *A* and *B* and S3 *A* and *B*). To further confirm that let-7adf directly targets Tet2, we engineered a luciferase gene with let-7–binding sites in its 3′-UTR and observed a significantly decreased luciferase activity when overexpressing let-7d via a let-7d mimic (Fig. 1*E*).

Collectively, these results demonstrate that Tet2 is an authentic target of let-7adf in LPS-activated macrophages, suggesting that let-7adf might promote IL-6 secretion by LPS-activated macrophages through repressing Tet2.

Let-7adf Is a Physiological Activator of IL-6 Production in Macrophages. To examine whether the let-7adf cluster regulates IL-6 production, we used LPS to activate cultured BMDMs derived from mice harboring a deletion of the let-7adf cluster and compared these cells to WT BMDMs. The IL-6 mRNA level following a 24-h stimulation with LPS was reduced by 50% in the absence of the

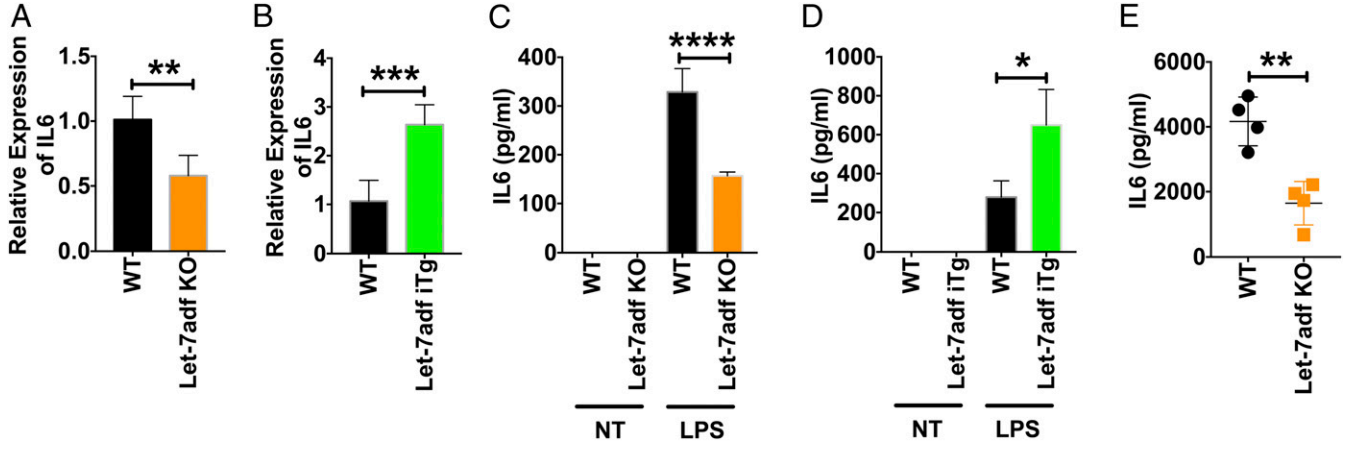

Fig. 2. Let-7adf is physiologically essential for IL-6 production in macrophages. (*A* and *C*) The let-7adf cluster KO/WT BMDMs were stimulated with LPS for 24 h. The IL-6 mRNA level was examined by qPCR (*A*); ELISAs were performed to examine the production of IL-6 in the cell culture supernatants of the let-7adf cluster KO/WT macrophages (*C*). (*B* and *D*) The let-7adf cluster iTg/WT BMDMs were stimulated with LPS for 24 h. The IL-6 mRNA level was examined by qPCR (*B*); ELISAs were performed to examine the productions of IL-6 in the cell culture supernatants of the let-7adf cluster iTg/WT BMDMs (*D*), and the BMDMs not treated with LPS served as controls (Ctrl). (*E*) The let-7adf cluster KO and WT mice were i.p. infected with sublethal dose of *Salmonella* and the IL-6 production in serum was assessed at 24 hpi. Each symbol represents one animal. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, using a Student's *t* test.

miR cluster (Fig. 2*A*). As expected, we observed the converse result with cells overproducing let-7adf: These cells displayed increased IL-6 mRNA following 24 h of LPS treatment (Fig. 2*B*). Similarly, IL-6 protein levels were decreased by deletion of let-7adf and were increased by overexpression of let-7adf (Fig. 2 *C* and *D*). These results demonstrate that let-7adf promotes IL-6 mRNA and protein production in activated macrophages in vitro.

To investigate the in vivo physiological consequences of manipulating let-7adf, we turned to a *Salmonella* bacterial infection model. When let-7adf KO and WT control mice were challenged with a sublethal dose of *Salmonella*, the let-7adf KO mice displayed significantly lower titers of IL-6 in their sera, compared with control WT mice, as evidenced by ELISA (Fig. 2*E*). Thus, let-7adf action contributes to IL-6 production against *Salmonella* infection in vivo.

It is evident that let-7adf is an important activator of macrophage IL-6 levels in vitro and in vivo.

Let-7adf Promotes IL-6 Through Repressing Tet2. To examine whether Tet2 is the mediator of the effects of let-7adf on IL-6 production, we asked whether deletion of Tet2 in macrophages had the opposite effect on IL-6 of deleting let-7adf. In fact, we observed that the absence of Tet2 led to a heightened IL-6 mRNA and protein production in LPS-activated macrophages in vitro (Fig. 3 *A* and *B*). Tet2 KO mice also showed consistent results, with higher IL-6 production in sera following LPS treatment compared with WT mice (Fig. 3*C*). These results indicate that loss of Tet2 mirrors the effect of let-7adf in macrophages on the regulation of IL-6.

To directly investigate whether Tet2 was involved in let-7adf–mediated IL-6 induction, an siRNA against Tet2 was utilized (*SI Appendix*, Fig. S4). We found that this siRNA, but not a scrambled version, could restore IL-6 mRNA and protein levels to normal levels, overriding the effects of let-7adf deletion (Fig. 3 *D* and *E*). Levels of IL-6 mRNA were significantly restored in the presence of siRNA-Tet2 compared with scrambled siRNA-treated let-7adf KO macrophages (Fig. 3*D*). The let-7adf KO macrophages transfected with scrambled siRNA did not show altered levels of mRNA expression for IL-6 compared with no treatment control (Fig. 3*D*). Consistently, we found that treatment of these cells with an siRNA-targeting Tet2 strongly rescued the impairment of LPS-induced IL-6 production by the absence of the miR cluster. We observed no IL-6 production without LPS treatment (Fig. 3E). In addition, Tet2 has been implicated in the late resolution phase of inflammation (6), so we examined the expression of Tet2/IL-6 over a time course to determine whether the regulation by let-7adf cluster is more widely modulating inflammation in activated macrophages. We found that IL-6 was lower expressed in let-7adf KO macrophages over the time course (*SI Appendix*, Fig. S5). The let-7adf KO macrophages showed higher Tet2 compared with WT controls at 4 h and 12 h (*SI Appendix*, Fig. S6). Next, we further asked whether the Let-7adf–mediated Tet2 regulation mode exists in other IL-6 inflammatory responses. We utilized another typical stimuli including TNFa, and we observed similar regulation pattern (*SI Appendix*, Fig. S7), indicating that the let-7adf–mediated Tet2 regulation mode also exists in TNFa-induced IL-6 inflammatory responses in macrophages.

Thus, it appears that the action of let-7adf on IL-6 is a consequence of the effect of let-7adf on Tet2, confirming that let-7adf promotes IL-6 expression through targeting Tet2 in LPS-activated macrophages.

Let-7adf Promotes Succinate Accumulation by Regulating Lin28a/Sdha Axis in Macrophages. We next sought to examine the metabolic consequences of let-7adf deletion in LPS-activated macrophages, examining first its possible role in LPS-induced glycolysis. BMDMs were treated with LPS, and the extracellular acidification rate (ECAR) was monitored (Fig. 4*A*). Let-7adf KO BMDMs showed a 50% lower extracellular acidification rate compared with WT BMDMs (Fig. 4*A*). Knowing that let-7adf contributes to LPS-driven glycolysis in LPS-activated macrophages, we tested whether let-7adf influenced mitochondrial function by measuring oxygen consumption rates (OCR). Remarkably, let-7adf KO BMDMs showed an even greater OCR curve than WT cells after LPS treatment (Fig. 4*B*), implying that let-7adf limits LPS-induced mitochondrial respiration in WT cells.

In agreement with a role for let-7adf in LPS action, let-7adf deficiency dramatically reduced LPS-induced succinate accumulation (Fig. 4*C*), indicating that let-7adf promotes glycolysis as well as the accumulation of succinate in LPS-activated WT macrophages. By contrast, we observed a higher ECAR and succinate level, as well as a lower OCR curve in the let-7adf iTg BMDMs, compared with WT BMDMs (Fig. 4 *D*–*F*), further confirming the role of let-7adf in enhancing glycolysis and promoting succinate accumulation in LPS-activated macrophages. To examine the pathway by which let-7adf controlled succinate accumulation within macrophages, we examined its effect on succinate dehydrogenase subunit A (Sdha), an enzyme important for succinate accumulation. SDHA expression in LPS-treated BMDMs was further enhanced by let-7adf KO and decreased in let-7adf iTg BMDMs (Fig. 4*G*), compared with WT controls,

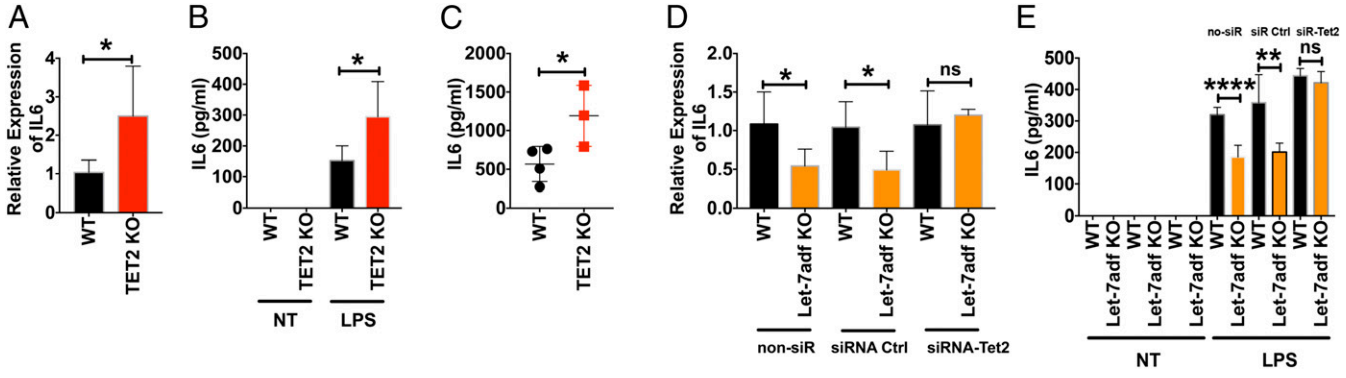

Fig. 3. Let-7adf promotes IL-6 by targeting Tet2. (*A* and *B*) The Tet2 KO/WT BMDMs were stimulated with LPS for 24 h. The IL-6 mRNA level was examined by qPCR (*A*); IL-6 production was examined by ELISA (*B*). (*C*) The TET2 KO and WT mice were injected with LPS (from *Salmonella*), and the IL-6 production in serum was assessed at 24 h after innoculation. (*D* and *E*) The BMDMs from let-7adf KO, WT, and KO treated with siRNA against Tet2 were stimulated with LPS for 24 h. The IL-6 mRNA level was examined by qPCR (*D*); the production of IL-6 in the cell culture supernatants of the Tet2 KO/WT BMDMs was examined by ELISA (*E*). ns, not significant; NT, no treatment; ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, using a Student's *t* test.

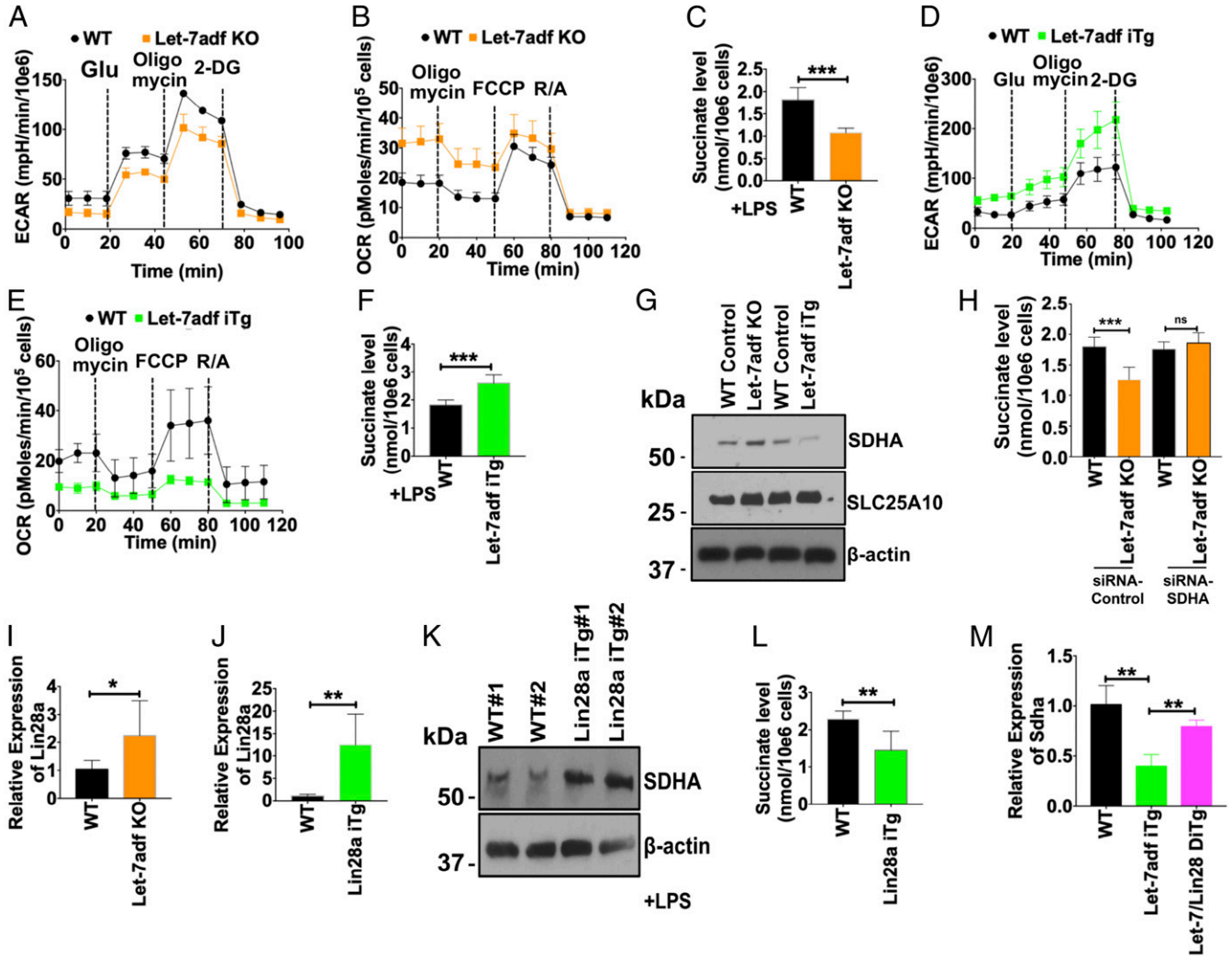

Fig. 4. Let-7adf promotes succinate accumulation by regulating the Lin28a/Sdha axis in macrophages. (*A*) The let-7adf KO/WT BMDMs were examined after 24 h culture following the LPS stimulation, and ECAR was determined by extracellular flux analysis. The representative plot of ECAR over time of macrophages with addition of glucose (10 mM), oligomycin (1 μM), and 2-DG (20 μM), as indicated. (*B*) The let-7adf cluster KO/WT macrophages were examined after 24-h culture following with LPS stimulation, and OCR was determined by extracellular flux analysis. Representative plot of OCR over time with addition of oligomycin (1 uM), mitochondrial uncoupler FCCP (0.5 μM), and electron transport inhibitors antimycin (1.5 μM) + rotenone (0.75 μM), as indicated. (*C*) Fold change of succinate accumulation in the LPS-activated let-7adf KO/WT BMDMs. (*D*) The let-7adf iTg/WT BMDMs were examined after 24-h culture following the LPS stimulation, and ECAR was determined by extracellular flux analysis. The representative plot of ECAR over time of macrophages with addition of glucose (10 mM), oligomycin (1 μM), and 2-DG (20 μM), as indicated. (*E*) The let-7adf cluster iTg/WT macrophages were examined after 24-h culture following with LPS stimulation, and OCR was determined by extracellular flux analysis. Representative plot of OCR over time with addition of oligomycin (1 μM), mitochondrial uncoupler FCCP (0.5 μM), and electron transport inhibitors antimycin (1.5 μM) + rotenone (0.75 μM), as indicated. (*F*) Fold change of succinate level in the LPS-activated let-7adf iTg/WT BMDMs. (*G*) The SDHA and SLC25A10 protein expressions in LPS-activated macrophages from the let-7adf KO/WT mice as well as the let-7adf iTg/WT mice obtained by Western blot. (*H*) Fold change of succinate level. Indicated siRNA-SDHA or siRNA control treated LPS-activated macrophages from let-7adf KO and WT mice were measured. (*I*) The Lin28a mRNA levels were analyzed by RT-qPCR in let-7adf KO/WT BMDMs. (*J*) The Lin28a mRNA levels were analyzed by RT-qPCR in Lin28a iTg/WT BMDMs. (*K*) The SDHA protein expressions in LPS-activated macrophages from the Lin28a iTg/WT mice obtained by Western blot. (*L*) Fold change of succinate level in the LPS-activated Lin28a iTg/WT BMDMs. (*M*) The Sdha mRNA levels were analyzed by RT-qPCR in LPS-activated let-7adf iTg, let-7adf/lin28a DiTg, and WT BMDMs. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, using a Student's *t* test.

suggesting that let-7adf inhibits SDHA protein expression in LPS-activated macrophages. We also tested whether let-7adf affected succinate transport by regulating SLC25A10, a key succinate transporter, but we found that SLC25A10 expression was comparable in let-7adf KO and WT control as well as in let-7adf iTg and control BMDMs (Fig. 4*G*).

To further test whether SDHA was involved in let-7adf–mediated succinate accumulation, an siRNA against SDHA was utilized. Treatment with this siRNA led to increased succinate accumulation in both KO and WT macrophages, which were now indistinguishable (Fig. 4*H*). Thus, our data indicate that SDHA works downstream of let-7adf and controls the succinate accumulation in macrophages.

To further investigate the molecular pathway directly connecting let-7adf to succinate accumulation mediated by Sdha, we explored the expression of lin28a, which is a well-established direct target of let-7 (18, 19). We found the lin28a mRNA expression was enhanced in let-7adf KO macrophages compared with WT control (Fig. 4*I*). We next utilized the engineered mouse model we had generated, which specifically overexpresses lin28a (denoted as Lin28a iTg mice) in macrophages about 20-fold (Fig. 4*J*). We found SDHA protein expression was higher in

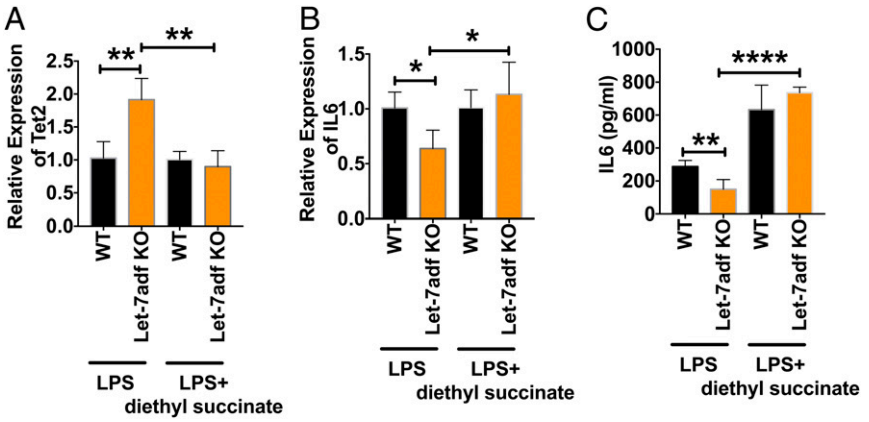

Fig. 5. Succinate contributes to another regulatory layer of let-7adf on Tet2 and promotes IL-6. (*A–C*) The let-7adf cluster KO, WT, and KO$^+$ diethyl succinate (pretreated for 2 h with 5 mM diethyl succinate) BMDMs were stimulated with LPS for 24 h. The Tet2 mRNA level was examined by qPCR (*A*); the IL6 mRNA level was examined by qPCR (*B*); the ELISAs were performed to examine the productions of IL-6 in the cell culture supernatants the let-7adf cluster KO, WT, and KO$^+$ diethyl succinate BMDMs (*C*). ****$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, using a Student's *t* test.

lin28a iTg macrophages, suggesting lin28a can also bind and enhance the accumulation of Sdha in macrophages (Fig. 4*K*), which is consistent with a previous report (20). Moreover, we observed a decreased succinate level in lin28a iTg macrophages, compared with a WT control (Fig. 4*L*), indicating that lin28a is one of the direct targets of let-7adf in inhibiting Sdha accumulation and enhancing succinate levels in LPS-stimulated macrophages.

To further validate whether lin28a mediates the effects of let-7adf in regulating Sdha in macrophages, we next utilized another engineered mouse model we had generated, which specifically overexpresses lin28a in let-7adf iTg mice (denoted as Let-7adf/Lin28a DiTg mice). We observed that let-7adf/lin28a DiTg macrophages showed enhanced Sdha expression, compared with the let-7adf iTg macrophages, suggesting that Lin28a overexpression partially rescued the altered Sdha expression regulated by let-7adf iTg (Fig. 4*M*).

Succinate Contributes to Another Regulatory Layer of Let-7adf on Tet2 and Promotes IL-6. Succinate, a proinflammatory metabolite, accumulates during LPS-induced macrophage activation, causing an increase in at least one proinflammatory cytokine, IL-1β (21, 22). Moreover, it has been reported that succinate can inhibit 2-oxoglutarate–dependent dioxygenases, including TET proteins (13, 14), raising the question of whether let-7adf regulates Tet2 through modulating succinate accumulation. In addition, our work has shown that let-7adf promotes succinate accumulation in macrophages by inhibiting SDHA, prompting us to examine the effects of succinate in LPS-activated macrophages. Using pretreatment of cells with the cell-permeable compound diethyl succinate, which increases succinate in the cytosol and mitochondrial matrix (22), we found that diethyl succinate significantly reduced the increased Tet2 level in let-7adf KO macrophages (Fig. 5*A*). This suggests that let-7adf acts both directly on Tet2 mRNA and indirectly through the accumulation of succinate to mediate Tet2 repression. Consistent with this notion, the decreased IL-6 mRNA and protein production in the let-7adf KO BMDMs were also overridden by addition of diethyl succinate (Fig. 5 *B* and *C*). Tet2 KO macrophages showed mildly changed levels of succinate accumulation (*SI Appendix*, Fig. S8), indicating that Tet2 is not essential to succinate production in LPS-activated macrophages.

Discussion

This study provides evidence of posttranscriptional regulation of Tet2 gene expression by the let-7adf miR cluster in LPS-activated macrophages. Tet2, but not Tet3, is a direct target of the let-7adf cluster. By inhibiting Tet2 production, the miR cluster potentiates IL-6 production in macrophages. Another consequence of the action of the let-7adf cluster on Tet2 is potentiation of succinate production, and succinate also promotes IL-6 activation. Our results imply a model (Fig. 6) by which the let-7adf cluster in LPS-activated macrophages regulates Tet2 through two pathways, one a direct effect on Tet2 mRNA and the other an indirect consequence of succinate regulation. Presumably, the effects on Tet2 levels caused by let-7adf and succinate cause alterations in DNA methylation, the only known effect of Tet2 (23, 24). Perhaps they occur at specific sites, but the specificity of Tet2 is ill-defined. How methylation alteration might lead to effects on cytokine gene transcription remains speculative.

Recently, endogenous metabolites including itaconate and succinate have emerged as key regulators of macrophage function, but their precise regulatory mechanism of action remains unclear. Succinate is clearly another key signaling molecule in innate immune cells (25, 26). Our current work links succinate to proinflammatory cytokine IL-6 secretion in LPS-activated macrophages. It will be important to know how succinate is transported and accumulates in activated macrophages, so that it triggers inflammation and innate immunity. Moreover, other key metabolites, such as α-ketoglutarate (α-KG), may contribute to

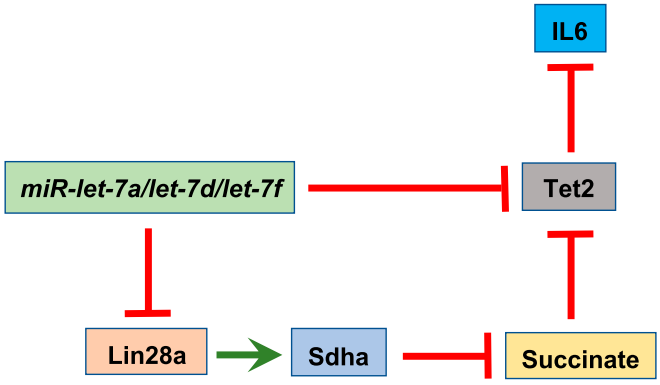

Fig. 6. The graphical abstract of dual mechanisms of Tet2 regulation by let-7adf in macrophages. The schematic diagram of the effects of let-7adf cluster on regulating Tet2 via two distinct mechanisms in macrophages: (*i*) through directly targeting Tet2; and (*ii*) via promoting succinate accumulation by regulating the Lin28a/Sdha axis.

Tet2 regulation in activated macrophages, because Tet2 utilizes α-KG as a substrate to hydroxylate 5mc to 5hmc during DNA demethylation (4). 2-Hydroxyglutarate (2-HG), which is produced by malfunction of isocitrate dehydrogenase (IDH) 1 or 2, competitively inhibits the function of α-KG–dependent enzymes such as Tet2 (27). Thus, to regulate Tet2 through targeting IDH may be another potential way to potentiate certain macrophage inflammatory responses. Interestingly, *Idh2* gene has a putative binding site for let-7 in its 3′UTR. It is possible that let-7adf might target *Idh2* in activated macrophages, resulting in another layer of regulation of Tet2, in turn, altering IL-6. IL-6 is a versatile regulator of physiology and pathological diseases (28, 29), and proper IL-6 expression is strictly controlled by post-transcriptional regulation (30–32). Our findings imply that modulation of succinate accumulation through variation of the let-7adf miR in activated macrophages could play a role in controlling IL-6. Our current study significantly added this miR let-7 cluster to one of the contributors that enhance IL-6 level in vivo. Our results suggest that the let-7adf cluster is an important inflammatory regulator of LPS-induced innate immune response in macrophages.

Materials and Methods

Mice. Tet2 KO and LysM Cre mice were purchased from The Jackson Laboratory. Lin28a LysM iTg and Let-7adf cluster LysM iTg mice were generated by crossing Lin28a iTg and Let-7adf iTg, with LysM-Cre, respectively. Let-7adf/Lin28a LysM DiTg mice were generated by crossing Lin28a iTg, Let-7adf iTg, and LysM-Cre mice. All of the mice were housed in the animal facility of California Institute of Technology. All of the animal procedures were carried out in accordance with Institutional Animal Care and Use Committee guidelines of California Institute of Technology.

Bacteria Strain and Infections. Bacteria used in this study were *Salmonella typhimurium* SL-1344 strain (*Salmonella*). Bacteria were grown to mid-logarithmic phase, pelleted, washed three times using PBS, and stored at −80 °C in small aliquots until use. The concentration of *Salmonella* was determined according to a standard growth curve based on the measurements of absorbance at 600 nm. In vivo *Salmonella* infections: 6–12 wk mice were infected intraperitoneally (i.p) with *Salmonella* SL-1344 strain. Sera were collected 24 h after *Salmonella* infections.

ELISAs Assay. ELISAs were performed according to manufacturers' instructions. Supernatants derived from the cultures of untreated or LPS-activated BMDMs were analyzed for IL-6 level with kits from eBioscience and carried out according to the manufacturer's instructions.

Statistical Analysis. All statistical analysis was done in GraphPad Prism software using an unpaired Student's *t* test. Data were reported as mean ± SEM. Significance measurements were marked as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, or ns for not significant.

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1. L. B. Ivashkiv, Epigenetic regulation of macrophage polarization and function. *Trends Immunol.* **34**, 216–223 (2013).
2. S. Wallner *et al.*, Epigenetic dynamics of monocyte-to-macrophage differentiation. *Epigenet. Chromatin* **9**, 33 (2016).
3. M. Ko *et al.*, Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 14566–14571 (2011).
4. X. Wu, Y. Zhang, TET-mediated active DNA demethylation: Mechanism, function and beyond. *Nat. Rev. Genet.* **18**, 517–534 (2017).
5. E. M. Kallin *et al.*, Tet2 facilitates the derepression of myeloid target genes during CEBPα-induced transdifferentiation of pre-B cells. *Mol. Cell* **48**, 266–276 (2012).
6. Q. Zhang *et al.*, Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. *Nature* **525**, 389–393 (2015).
7. J. J. Fuster *et al.*, Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science* **355**, 842–847 (2017).
8. R. M. O'Connell, D. S. Rao, D. Baltimore, microRNA regulation of inflammatory responses. *Annu. Rev. Immunol.* **30**, 295–312 (2012).
9. L. F. Zhang, S. Jiang, M. F. Liu, MicroRNA regulation and analytical methods in cancer cell metabolism. *Cell. Mol. Life Sci.* **74**, 2929–2941 (2017).
10. L. Wu *et al.*, Precise let-7 expression levels balance organ regeneration against tumor suppression. *eLife* **4**, e09431 (2015).
11. B. B. Madison *et al.*, LIN28B promotes growth and tumorigenesis of the intestinal epithelium via Let-7. *Genes Dev.* **27**, 2233–2245 (2013).
12. S. Jiang, W. Yan, S. E. Wang, D. Baltimore, Let-7 suppresses B cell activation through restricting the availability of necessary nutrients. *Cell Metab* **27**, 393–403.e4 (2018).
13. W. A. Pastor, L. Aravind, A. Rao, TETonic shift: Biological roles of TET proteins in DNA demethylation and transcription. *Nat. Rev. Mol. Cell Biol.* **14**, 341–356 (2013).
14. C. Zhong, J. Zhu, Tet2: Breaking down barriers to T cell cytokine expression. *Immunity* **42**, 593–595 (2015).
15. T. Laukka *et al.*, Fumarate and succinate regulate expression of hypoxia-inducible genes via TET enzymes. *J. Biol. Chem.* **291**, 4256–4265 (2016).
16. E. Letouzé *et al.*, SDH mutations establish a hypermethylator phenotype in paraganglioma. *Cancer Cell* **23**, 739–752 (2013).
17. D. Betel, A. Koppal, P. Agius, C. Sander, C. Leslie, Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90 (2010).
18. S. Jiang, D. Baltimore, RNA-binding protein Lin28 in cancer and immunity. *Cancer Lett.* **375**, 108–113 (2016).
19. N. Shyh-Chang, G. Q. Daley, Lin28: Primal regulator of growth and metabolism in stem cells. *Cell Stem Cell* **12**, 395–406 (2013).
20. N. Shyh-Chang *et al.*, Lin28 enhances tissue repair by reprogramming cellular metabolism. *Cell* **155**, 778–792 (2013).
21. G. M. Tannahill *et al.*, Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* **496**, 238–242 (2013).
22. E. L. Mills *et al.*, Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell* **167**, 457–470.e13 (2016).
23. K. D. Rasmussen, K. Helin, Role of TET enzymes in DNA methylation, development, and cancer. *Genes Dev.* **30**, 733–750 (2016).
24. E. Solary, O. A. Bernard, A. Tefferi, F. Fuks, W. Vainchenker, The Ten-Eleven Translocation-2 (TET2) gene in hematopoiesis and hematopoietic diseases. *Leukemia* **28**, 485–496 (2014).
25. F. Ginhoux, S. Jung, Monocytes and macrophages: Developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* **14**, 392–404 (2014).
26. B. Kelly, L. A. O'Neill, Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* **25**, 771–784 (2015).
27. D. Ye, K. L. Guan, Y. Xiong, Metabolism, activity, and targeting of D- and L-2-hydroxyglutarates. *Trends Cancer* **4**, 151–165 (2018).
28. T. Tanaka, M. Narazaki, K. Masuda, T. Kishimoto, Regulation of IL-6 in immunity and diseases. *Adv. Exp. Med. Biol.* **941**, 79–88 (2016).
29. J. Mauer, J. L. Denson, J. C. Brüning, Versatile functions for IL-6 in metabolism and cancer. *Trends Immunol.* **36**, 92–101 (2015).
30. J. L. Zhao, D. S. Rao, R. M. O'Connell, Y. Garcia-Flores, D. Baltimore, MicroRNA-146a acts as a guardian of the quality and longevity of hematopoietic stem cells in mice. *eLife* **2**, e00537 (2013).
31. J. L. Zhao *et al.*, Conversion of danger signals into cytokine signals by hematopoietic stem and progenitor cells for regulation of stress-induced hematopoiesis. *Cell Stem Cell* **14**, 445–459 (2014).
32. C. A. Hunter, S. A. Jones, IL-6 as a keystone cytokine in health and disease. *Nat. Immunol.* **16**, 448–457 (2015).